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Evaluation of the Antibacterial Properties of *Lactobacillus acidophilus* Metabolites against Oral Plaque Streptococci: An *In vitro* Study

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ABSTRACT

The bacterial plaque is one of the important factors in the destruction of teeth and periodontal tissues. Probiotics have been proved to be effective in the plaque reduction and gingival health maintenance. Lactic Acid Bacteria (LAB), especially lactobacilli are known for their antimicrobial activity. Most of the antimicrobial activity is due to the secondary metabolites produced by certain LAB strains. The aim of this study was to investigate the antibacterial effects of Lactobacillus acidophilus metabolites on common oral streptococci. In this in vitro investigation, the minimum inhibitory concentrations (MIC) of Lactobacillus acidophilus metabolites was assessed using a modified E test, against three plaque-forming bacteria, including Streptococcus mutans, Streptococcus sanguinis, and Streptococcus salivarius. The metabolites of Lactobacillus acidophilus were extracted using ethyl acetate. The L. acidophilus metabolites showed potent inhibitory activity against all tested strains. The MICs for S. mutans, S. sanguinis, and S. salivarius were 0.01875, 0.009 and 0.15 mg/ml, respectively. The findings of this study revealed that L. acidophilus produced compounds with a good antibacterial activity which may provide a basis for alternative therapies for the prevention and control of the oral plaque-forming bacteria.

Key words: *Lactobacillus acidophilus*, Probiotics, Oral plaque, Antibacterial properties

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INTRODUCTION

The use of probiotics to increase and improve the human health is a long-standing proposal. In recent years, many studies have been conducted to confirm the anti-pathogenic effects of probiotics. Probiotics are defined as viable microorganisms that are beneficial to their host when administered in adequate amounts [1,2]. Some of the bacterial genera most used in probiotic preparations include *Bifidobacterium, Lactobacillus, Saccharomyces* and *Leuconostoc* [3]. The *Lactobacillus* genus contains various Gram-positive facultative anaerobic or microaerophilic rod-shaped microorganisms. They are a major part of the lactic acid bacteria (LAB) group. Lactic acid bacteria (LAB) are among the probiotics that produce various antimicrobials components and bacteriocins. Due to their antimicrobial properties, probiotics have been suggested as prophylactic and therapeutic agents for oral diseases caused by bacteria found in dental plaque [3-7].

Dental plaque is a complex of microbial community found on the surface of teeth, embedded in a matrix of bacterial and salivary origin [8]. It is obvious that the specific nature of the microbiota of dental plaque is fundamental in the etiology and pathogenesis of dental caries and periodontitis [6,8]. Recently, Preventive approaches based upon the use of probiotic bacteria to reduce antibiotic use and develop novel treatments for oral diseases that do not involve conventional antimicrobial agents have been proposed [7,9].

Lactobacillus acidophilus, a member of the normal intestinal and vaginal flora of healthy humans, is one

of the most commonly suggested *Lactobacillus* species for use as a probiotic [10,11]. Some researchers from developed countries have reported significant *in vitro* inhibition of Gram negative and Gram-positive pathogenic bacteria by *Lactobacillus acidophilus* species, but similar studies and information about the inhibitory effects of *Lactobacillus acidophilus* against oral *streptococci*, which plays an important role in the development of dental plaques, from developing countries, such as Iran, are very limited [12,13].

Therefore, the aim of this study was to investigate the *in vitro* inhibitory effects of *Lactobacillus acidophilus* metabolites against the main plaque-forming streptococci including *S. mutans, S. sanguinis,* and *S. salivarius.*

MATERIALS AND METHODS

Ethics

The study was approved by the Research Ethics Committee of the Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (No: IR.AJUMS. REC.1397.079).

Strains and culture conditions

This study was conducted in the Microbiology Laboratory of the Department of Microbiology of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran, during January to May 2018. All standard strains of *Streptococcus mutans* (PTCC 1683), *S. sanguinis* (PTCC 1449), *S. salivarius* (PTCC 1448) and *L. acidophilus* (PTCC 1643) were purchased from the Persian Type Culture Collection (PTCC) of the Iranian Research Organization for Science and Technology (IROST) in Tehran, Iran and cultured according to the manufacturer's instructions. *L. acidophilus* was cultured in MRS (De Man, Rogosa, and Sharpe) broth (Merck, Darmstadt, Germany) for 48 hours under a 5% CO_2 atmosphere at 37°C. Streptococci strains were cultured on TSB (Triptic Soy Broth) (Merck, Darmstadt, Germany) with 5% defibrinated sheep blood and incubated for 24 h to 48 h at 37°C under aerobic conditions. The physiological features and purity of the isolates were analyzed by observation of their morphological characteristics using a light microscope, Gram staining, and a catalase test. Stock cultures of all strains were maintained at −20°C in 15% glycerol.

Extraction of Lactobacillus acidophilus metabolites

The colony of *L. acidophilus*, inoculated in MRS broth. After 3 days incubation, the MRS broth media containing bacteria was mixed with ethyl acetate (75:25) and agitated with a magnetic stirrer for 8 h. Then the media was allowed to settle for 30 min. Following the settlement, the solution was separated into two phases, which the supernatant was comprised of the extracted antimicrobial compound. The upper organic layer was separated using a separating funnel and centrifuged at 5000 rpm for 10 minutes. The ethyl acetate layer was then removed and transferred into a clean flask. The extract was pooled and dried in an incubator at 45°C. The yield from the extract was dissolved in methanol (Merck, Germany) and its pH was adjusted to 7 using NaOH for antimicrobial susceptibility testing [10,14].

Antimicrobial susceptibility test (AST)

Antibacterial activity on plates was done by the disc diffusion method (DDM). Initially, a bacterial suspensions equivalent to 0.5 McFarland standard inoculum were prepared and then inoculated onto Mueller-Hinton agar (MHA) (Merck, Germany) containing 5% sheep blood. Subsequently, 10 µl of the extract was loaded on to a sterilized 6 mm diameter Whatman no. 1 filter paper disc, and dried in sterile Petri dish at 37°C for 10 min. The dried discs were placed on the bacterial growth and diameter of inhibition zone (DIZ) around each disc was measured after 24 h incubation at 37°C. The antagonistic activity of the *L. acidophilus* was measured in triplicates and the data have been presented in Table 1.

The minimal inhibitory concentration (MIC) of *L. acidophilus* metabolite substance determined using an improved modified E test method (AB Biodisk, Solna, Sweden) in order to show the antimicrobial activity of *L. acidophilus* [10,14,15]. In the improved E test, several AB Biodiscs impregnated with different dilutions of the extracts were used instead of strips. In fact, it was a simulated version of the standard E test [15]. The *S. mutans, S. sanguinis* and *S. salivarius* suspensions of freshly grown cultures were adjusted to a density of 10^6 cells/mL, corresponding to 68%-82% transmittance at 530 nm. The plate of Mueller-Hinton agar (MHA) with 5% sheep blood was inoculated by dipping a sterile cotton swab into the suspension and streaking it across the agar surface in three directions. The plates were dried at an ambient temperature for 15 min before applying the discs. Six sterile discs (6 mm) were impregnated with 10 µl of six different concentrations (0.15, 0.075, 0.0375, 0.01875, 0.009 and 0.004 mg/ml) of the serially diluted metabolite in methanol and placed in a line on the agar surface. The plates were incubated for 24 h at 37°C. The MIC values were read as the antimicrobial concentrations at the points where dense colonial growth intersected the discs [15]. The test was performed in triplicate for each strain.

RESULTS

The antimicrobial activity of the extracted metabolites from *L. acidophilus* (PTCC 1643) strain was assessed against three oral *streptococci* in Table 1. The DDM test showed the largest zone of inhibition (18 mm) against the *Streptococcus sanguinis*. The least zone of inhibition, 8 mm was shown against *S. Salivarius*. The findings of the DDM and modified E test showed a potent inhibitory activity of the *L. acidophilus* metabolites against all tested strains in Figure 1. The MICs of metabolites compound extracted from *L. acidophilus* were 0.01875, 0.009, and

Table 1: Results of disc diffusion test of *Lactobacillus acidophilus* metabolite against indicator strains

Indicator Strain	DIZ
Streptococcus mutans	12
Streptococcus sanguinis	18
Streptococcus salivarius	8

Note: Values refer to the diameter of inhibition zone (in mm) and represent mean (average) of triplicates

0.15 mg/ml for *S. mutans, S. sanguinis,* and *S. salivarius*, respectively (Table 2). The metabolite extract exhibited the greatest effect on *S. Sanguinis*. Among the tested strains, *S. salivarius* showed the highest resistance to metabolites extract.

DISCUSSION

The present study aimed to determine whether the *L. acidophilus* (PTCC 1643) metabolites were able to exert an antagonistic effect against oral *streptococci* by disc diffusion and modified E test methods. *In vitro* experiment has revealed that selected *L. acidophilus* strain is effective against *oral streptococci*. Previous studies from different countries have been shown the important role of *lactobacilli* as potential antibacterial agents against pathogens such as *Enterococcus faecium, Shigella sonnei* and *Clostridium perfringens* [12,13,15].

Furthermore, *Lactobacillus acidophilus* is among the various *lactobacilli* that have been studied for their antibacterial effects in previous investigations [16]. Mohankumar et al. claimed that *L. acidophilus* had antagonist effects on the growth of *Proteus* spp. that is associated to the bacteriocin production [17]. The microbial inhibitory activity of *lactobacilli* can be probably attributed to the production of several antimicrobial compounds like bacteriocin, organic acid and hydrogen peroxide [18]. Dental caries and periodontal diseases are a major public health challenge in all countries of the world. The oral *streptococci* such as *Streptococcus mutans* play the major role in dental caries and periodontal diseases pathogenicity [6,7].

Because of the increasing antibiotic resistance of bacteria, new methods such as probiotic therapy for decreasing of oral cavity pathogens must be investigated. *Lactobacillus acidophilus* was investigated in this study because of its known probiotic potential [11]. According to our results, it is cleared that the (PTCC 1643) can inhibit the growth of *streptococcal* strains. Our study confirmed the results obtained by Khanafari et al. in whose study *L. acidophilus* (PTCC 1643) had the inhibitory effect on *S. mutans* isolated from samples of gingival dental plaque and caries lesions in adults [19]. Regarding the *L. acidophilus* antibacterial properties, research conducted by Tahmourespour et al. showed that *L. acidophilus* was able to reduce the plaque formation of oral *Streptococci* [20]. In this study, the metabolite extract of *L. acidophilus* showed the best inhibitory effect on *S. Sanguinis.* Consistent with our findings, Miller et al. reported that the addition of the *lactobacilli* to cultures of *Streptococcus sanguinis* resulted in more inhibition of plaque formation when compared with pure cultures of *Streptococcus sanguinis* [21]. A survey by Aween et al. aimed at studying the antibacterial activity of *L. acidophilus* strains isolated from honey marketed in Malaysia, showed inhibitory activity against multiple antibiotic resistant's *Staphylococcus aureus, Staphylococcus epidermis,* and *Bacillus subtilis* in the agar overlay method after 24 h incubation at 30°C [22]. In another study, Abdulla investigated the antimicrobial activity of *L. acidophilus* and revealed the highest inhibitory activity against *B. subtilis, Pseudomonas aerogenosa, Streptococcus pyogenes, Proteus vulgaris, Staphylococcus aureas,* and *Aeromonas hydrophila* [23]. Mahmood et al. reported that different strains of L. acidophilus cultured from a native milk product exerted inhibitory effects against food borne pathogens [24]. Jabbari et al. reported that the *L. acidophilus* isolated from traditional doogh samples which collected from the Tabriz region, Iran had a growth inhibitory activity against *Staphylococcus aureus, Shigella dysenteriae,* and *Escherchia coli* [25]. Because of the therapeutic potentiality of probiotic *lactobacilli* against MDR bacterial infection, they are one such microorganisms of choice to use as the remedial agents in combination with antibiotics [26]. As has been reported by Bassyouni et al. the *L. acidophilus*-ciprofloxacin combined therapy against infective gastroenteritis was effective in terms of decreasing the ciprofloxacin (CIP) dose and hence the CIP adverse effect on the balance of microbial flora in human intestine [27].

Figure 1: Antimicrobial activity of *Lactobacillus acidophilus* (PTCC 1643) metabolite against Streptococcus mutans (PTCC 1683) by modified E test

Table 2: Results of MICs of *Lactobacillus acidophilus* metabolite against indicator strains

Indicator Strain	MIC (mg/ml)
Streptococcus mutans	0.01875
Streptococcus sanguinis	0.009
Streptococcus salivarius	0.15

Several studies have been conducted on the antibacterial potential of *lactobacilli*, which showed various results. There are some dissimilarities and contradictions between the results, indicating that the efficacy of *lactobacilli* products is significantly affected by many factors such as the method of metabolite extraction, antibacterial assay method and conditions, and the source of tested strain. In the present study, ethyl acetate was used to obtain the *Lactobacillu*s extract. In other studies, researchers used the filtered supernatant fluids from MRS liquid medium containing *Lactobacillus* cells as antimicrobial extract [15]. In our study, Sodium hydroxide (NaOH) was added to the extract in order to neutralize its pH. Therefore, the antimicrobial activity of the extract was not related to its acidic nature.

However, the results obtained in this study can serve a guide for further clinical investigations, in view of the limitations of *in vitro* studies, *in vivo* survey and further research using more complex techniques are needed to support the efficacy of *L. acidophilus* as an antibacterial probiotic against cariogenic pathogens

CONCLUSION

This work highlights the antimicrobial potential of *L. acidophilus* metabolite *in vitro*. The results of the study allow us to suggest further research to introduce *L. acidophilus* for use in the prevention and control of dental plaque formation and other dental diseases.

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CONFLICT OF INTEREST

The authors' declares that they have no conflict of interest.

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